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Full Length Article

ERα and ERK1/2 MAP kinase expression in microdissected stromal and epithelial endometrial cells

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Abstract Our previous published data detected higher expression of total and active mitogen activated protein kinase (MAPK) in the epithelial vs. stromal cells of the endometrium. In the present work we compared the expression of ERK1/2 MAPK and estrogen receptor α (ERα) in epithelial versus stromal cells in benign human endometrial tissues. Laser capture microdissection was used to separate glandular epithelium and stromal cells from six frozen, proliferative phase endometrial specimens.

Total and phosphorylated levels for ERK1/2 and ERα were measured by quantitation of signals from Western blots using specific antibodies against the active and total forms of ERK1/2 and against ERα. When the level of the proteins was quantitated and normalized to β actin from microdissected stroma and epithelium, no significant difference was detected in the levels of these proteins between the two tissue compartments. There was a trend toward higher expression in the stroma vs. epithelium, respectively (active ERK1/2 0.45 ± 0.17 vs. 0.2 ± 0.65; total ERK1/2 0.54 ± 0.35 vs. 0.28 ± 0.23; ERα 0.82 ± 0.28 vs. 0.54 ± 0.18; $n = 6$). These data demonstrate that there are comparable levels of ERα ($P = 0.41$), total ERK1/2 ($P = 0.18$) and active ERK1/2 ($P = 0.13$) in the stroma and epithelium of proliferative phase endometrium with a trend toward higher expression of these proteins in the stromal compartment.

Introduction

There is growing interest in studying the distribution of mitogen activated protein kinase (MAPK) in different tissue compartments to identify a target tissue compartment that would mediate the effects of novel signal transduction inhibitors that target the MAP kinases. Interference with MAP kinase signaling has the potential to benefit therapies targeting the estrogen receptor (ER) in the endometrium. The antiestrogen tamoxifen blocks ER function, which is the basis for tamoxifen use in breast cancer. However, tamoxifen exhibits estrogen-like

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action in the endometrium and tamoxifen use may be associated with abnormal endometrial proliferation that may progress to endometrial carcinoma. Experimental evidence has shown that the extracellular signal-regulated kinase 1 and 2 (ERK1/2) [P44/42 MAP kinase] pathway cross-talks to ER and promotes the estrogen-like action of tamoxifen most notably in estrogen dependent tumors [13,17]. This likely occurs through phosphorylation of ER as well as reciprocal activation of ERK1/2 by estrogens [21].

Interactions between stroma and epithelium play an important role in the growth and differentiation of endometrium [4]. ER-dependent proliferation may be mediated by direct action of estrogen on epithelial ER and/or an indirect effect through the stromal ER. Animal models suggest that the stroma directs the epithelial response to estrogen [6,5]. It was shown that stromal ER directs the epithelium to respond to estrogen with proliferation in neonatal mice [20]. In other studies where separated endometrial epithelial and stromal cells were recombined and grown in athymic mice, the ER positive stroma was important as an interpreter of the estrogenic proliferation signal to the glandular epithelium [15,27]. It is likely that estrogen regulates transcription in a gene specific manner through ER present in both stroma and epithelium [16,22].

Bouchard et al. reported intense and frequent glandular and stromal immunoreactivity of ER during the mid proliferative phase. During the mid and late secretory phase, ER immunoreactivity almost disappeared from the glandular epithelial cells [7]. Others found that the highest concentration of ER was detected in both epithelial and stromal cells during the late proliferative phase and decreased in the secretory phase [2,18,19,23].

In our previous study [9] we found elevated active and total ERK 1/2 in glandular epithelium in comparison to the stromal cells in proliferative phase endometrium using immunohistochemistry (IHC) in formalin-fixed paraffin embedded sections in three out of six cases used in the present study. Laser capture microdissection (LCM) offers a method to separate cell types from heterogeneous tissues using standard microscopic visualization for subsequent analysis. The selected cells are removed by laser pulses and captured on a thermoplastic cap where the captured cells can be visualized and display intact architecture. Distinct tissue morphology must be maintained by proper trimming and staining to accurately separate the cells from frozen sections most notably because the sections are relatively thick and no glass coverslip will be applied to the sections [24]. Other methods used to separate tissues such as enzymatic techniques employing 1% trypsin followed by mechanical manipulations do not allow for completely accurate separation of stroma and epithelium [4].

No studies have examined the quantitative expression of ER or ERK1/2 kinase from glandular endometrial epithelium and stroma cells separately excised by LCM. This approach provides a mean to accurately quantitate and compare expression levels of these proteins among the tissue compartments.

Materials and methods

Six freshly frozen, human endometrial tissues that showed higher expression of ERK 1/2 by Western blot in our previous study [9] were obtained from the Cooperative Human Tissue Network (CHTN, Columbus, OH). The histological type was normal mid proliferative phase endometrium. The tissues were immediately snap frozen in liquid nitrogen after resection to minimize protein degradation and dephosphorylation of the kinases. Each endometrial tissue (0.2–1.5 g) was cut into four pieces and one of them was embedded in M1 embedding matrix for frozen sectioning. The tissue was placed in an empty cryomold on dry ice, and M1 matrix was poured into the cryomold. The blocks were kept at −80 °C until sectioning. Cyrosections were prepared (8 μm) (Leica CM 3050 S cryostate, Meyer Instruments, Inc., Houston, TX) on Poly-L-lysine glass slides, placed on dry ice and stored at −80 °C until LCM. One section was used at a time, fixed with 70% ethanol at 4 °C for 60 s, followed by deionized water for 30 s., staining with Nuclear Fast Red (NFR) containing protease inhibitor cocktail (150 μl of Sigma protease inhibitor cocktail in 10 ml of stain) for 60 s, followed by 2 × 2 min dH2O. The slide was dehydrated with successive washes of 70%, 95% and 100% ethanol for 30 s. each, xylene 2 × 5 min. and air dried for 15 min.

Laser capture microdissection

The slides were microdissected using an Arcturus PixCell II system (PixCell 100; Arcturus, Mountain View, CA) to separate the endometrial glandular epithelium from the stromal tissue with careful histological evaluation by the study pathologist (MMD). For each case, 25,000–35,000 laser pulses were used with the amplitude and pulse duration fixed at 45 mW for 30 ms and the diameter of the laser beam adjusted to 7.5 μm. The microdissected tissue was adhered to caps (CapSure LCM Caps, Arcturus, Mountain View, CA). The caps were inverted in matching microfuge tubes containing 50 μl of lysis buffer [10 mM Tris pH 8, 0.4 M sodium chloride, 50 mM potassium phosphate, 50 mM sodium fluoride, 10 mM sodium molybdate, 2 mM EDTA, 2 mM EGTA, 1 mM sodium vanadate, 0.1% triton-X 100, 10 mM β mercaptoethanol, 0.1% SDS, 0.5% IGEPAL CA-630, protease inhibitor cocktail (Sigma, St. Louis, MO) and 0.1 mM phenylmethyl-sulfonyl fluoride (PMSF)]. Proteins were extracted by brief vortexing of the tube and centrifugation at 20,000×*g* in a microfuge to clear the supernatant. 12.5 μl of 5× SDS puffer was added to the cleared supernatant and the sample was stored at −20 °C until use.

Western blot analysis

Polyclonal anti-rabbit antibodies against total and phosphorylated (active) kinases for human p42/44 MAP kinase (ERK1/2), were purchased from Cell Signaling Technology, (Beverly, MA, USA), monoclonal anti-mouse antibody to estrogen receptor (NCL-ER-6F11) was purchased from Novo Castra, (Newcastle, UK), and antibody to β actin was purchased from Santa Cruz Biotechnology, (Santa Cruz, CA). Secondary antibodies (biotinylated anti-rabbit, anti-mouse and anti-goat IgG (H + L)), and horseradish peroxidase streptavidin were purchased from Vector Laboratories, (Burlingame, CA). Equal amounts of protein extraction were separated by electrophoresis on a 10% SDS–PAGE gel. The gel was transferred to a nitrocellulose membrane, and blocked in 5% nonfat milk in Tris-buffered saline/Tween 20 (TBST; 10 mM Tris, pH 8, 150 mM NaCl and 0.1% Tween-20) for 1 h at room temp., followed by 3 × 5 min. washes with TBST. The membranes were incubated with each primary antibody overnight in 5% dried milk/TBST at 4 °C at a 1:500 dilution for ERK 1/2 and a 1:40 dilution for the estrogen receptor antibody. Following

primary antibody incubation, the membranes were washed with TBST 3 × 5 min. and then incubated with secondary biotinylated IgG (3 μg/ml) for 1 h at room temp. Following 3 × 5 min washes with TBST, the membranes were incubated with horseradish peroxidase streptavidin (2 μg/ml) for 1 h at room temp., and washed 3 × 5 min. with TBST. The signal was developed with the addition of enhanced chemiluminescence (ECL) solution (1:1 mixture of solution A + B (A) 9.9 ml of 0.1 M Tris pH 8.5 + 100 μl luminol + 44 μl P-coumaric, (B) 10 ml of 0.1 M Tris pH 8.5 + 6 μl hydrogen peroxide). The membranes were exposed to Kodak Imager instrument for signal quantitation as described below and also exposed to a Kodak XOMAT film for 15 min. Samples that showed no Western blot signal were repeated two times to assure there were no problems with the Western blot procedure. To assess for protein degradation and to normalize protein load among samples, the membranes were incubated with antibody against β actin after stripping the blots (see below).

Stripping of membranes

After performing Western blotting using antibodies to the kinases and ER, the membranes were incubated in stripping buffer (62.5 mM Tris–HCl (pH 6.8), 2% SDS and 100 mM β-mercaptoethanol) for 30 min., at 55 °C with rocking to remove bound antibodies and prepare the membrane to be re-probed with antibody against β-actin (2 μg/ml) as described above.

Quantitation of the ECL signal

The ECL signal was quantitated using a Kodak Digital Science 1D image analysis software station 440 system (Kodak Digital Science, Rochester, NY) to compare the level of activated and total kinases and ERα between glandular epithelium and stroma cells for each protein separately among samples. The signals measured for these antibodies were divided by the value for β-actin to normalize for protein load.

Statistical analysis

SPSS software for Windows (version 11.5.1) was used in analyzing the Western blot data. Values were reported as means ± SEM. The Mann–Whitney *U* test was used to analyze the differences in the expression of total ERK1/2 and the independent *t*-test was used to analyze the differences in the expression of active ERK1/2 and ERα between glandular epithelium and stromal cells. $P \leqslant 0.05$ was considered significant.

Results

Our objective in the present study was to quantitatively compare the expression of active and total ERK 1/2 MAP kinase and ERα in laser captured microdissected benign proliferative glandular epithelium versus stromal cells from freshly frozen endometrial tissue samples after brief fixation in 70% ethanol and staining with NFR. All six cases studied were normal, mid proliferative phase endometrium (Fig. 1A and B). The glands were uniform, tubular, lined by one layer of columnar cells, showing frequent normal mitoses and surrounded by dense cellular stroma. The same fields are shown in Fig. 1C and D after LCM of the glandular epithelium but with NFR stain.

We observed that the tissue architecture was well preserved and that tissues were easily removed by LCM. From six specimens there was no significant difference in the level of active and total ERK1/2 and ERα between stroma and epithelium (Fig. 2) when the absolute Western blot signal of these antibodies was normalized to β actin [active ERK1/2 (0.45 ± 0.17 vs. 0.2 ± 0.65) total ERK1/2 (0.54 ± 0.35 vs. 0.28 ± 0.23) and ERα (0.82 ± 0.28 vs. 0.54 ± 0.18)] in stromal versus glandular epithelial cells, $P = 0.13$, 0.18 and 0.41, respectively. Similar results were found for active ERK1/2 when the signal was normalized to the total ERK1/2 signal rather than β actin (data not shown). However, there was a trend toward higher levels of these proteins expressed in stroma vs. epithelium with the increased expression of active ERK1/2 in the stroma approaching significance ($P = 0.13$). A larger sample size than the one used in this report may be used in future studies to determine if actual differences exist.

Discussion

In the present work we measured both total and active levels for ERK1/2 MAPK and ERα in glandular epithelium versus stromal cells from six freshly frozen, mid proliferative phase human endometrial tissue specimens that showed the highest expression of ERK 1/2 in our previous study by means of Western blot after tissue homogenization and total protein extraction.

In our previous IHC results, where three out of the six cases used in the present study were stained with the same antibodies for active and total ERK 1/2, we found an elevated active and total ERK 1/2 in the glandular epithelium compared to mild expression in the stromal compartment [9]. The ERα immuno-staining also appeared to be more intense in the epithelial compartment compared to the stromal compartment in the same cases studied (data not shown) giving the impression that the glandular epithelial expression of ERK 1/2 and ERα is higher than that in the stroma.

LCM offers an advantage over standard IHC by providing precise quantitative measurement of the expression of proteins in specific tissue sub-compartments with normalization to standard proteins [10,24]. In contrast to what we and others have found using IHC for ERα [2,9,18,19,23] we detected no significant difference in the expression of total ERK1/2, active ERK1/2 and ERα between stroma and epithelium, with a trend toward higher expression of these proteins in the stroma. Although the absolute quantitated values were higher in the glandular epithelium compared to the stroma, when normalized to β actin the differences were not significant. The relative expression of these proteins as assessed by IHC may be misleading since normalization to β actin is not performed during IHC.

Our work of quantitative measurement of stromal and epithelial ER using LCM confirms the significant stromal ERα expression when compared to the epithelium and supports a role for the stromal ERα. Yamashita and co-workers used IHC in neonatal CD-1 mouse uterus and found that the epithelial cells of the endometrium expressed ER but at low levels compared to the stroma [27,26]. Garcia et al. reported that ERα is expressed in both human endometrial glandular epithelium as well as stroma cells [14]. Cooke et al. reported that epithelial ERα is not necessary for estrogen induced epithelial proliferation and that the stromal component has the most important tissue compartment for proliferation [8]. Multiple reports suggest that the stromal ER is important for the

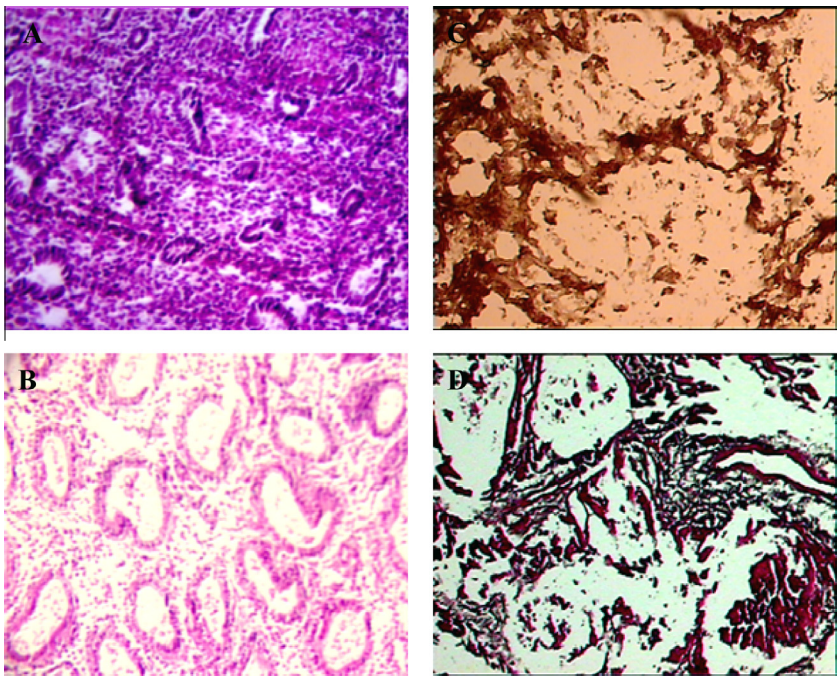

Figure 1 Representative cases of mid proliferative phase endometrial tissue sections used for laser capture microdissection. (A and B) Formalin fixed paraffin embedded sections stained with H&E showing uniform tubular glands lined by one layer of columnar cells surrounded by cellular stroma. (C and D) The same cases but cryo-sectioned and stained with NFR after a brief 70% alcohol fixation and subjected to LCM to separate the glandular epithelial cells from the stromal cells. Note the empty fields after extraction of the glands. Original magnifications are (×200).

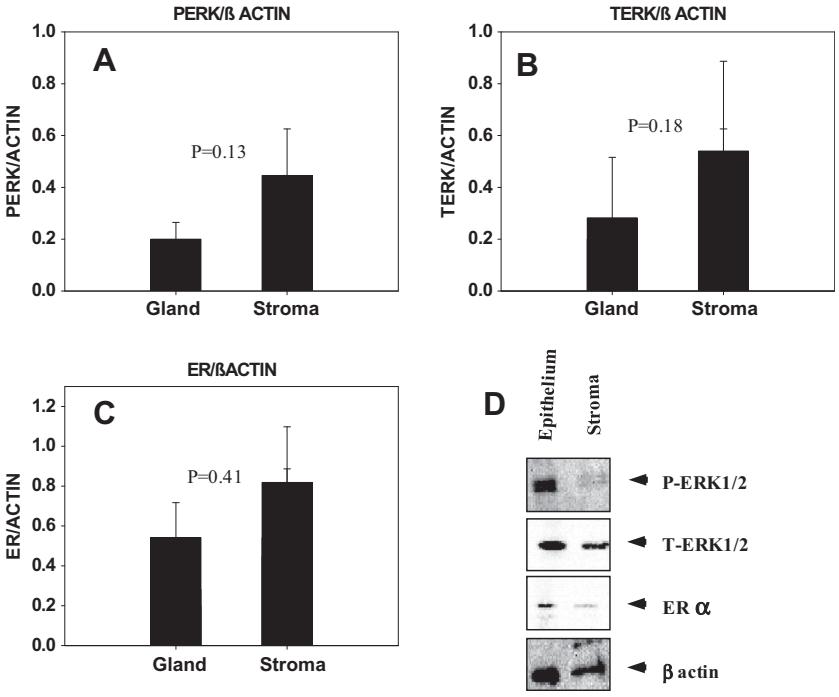

Figure 2 Expression of ERK1/2 kinase and ERα in microdissected endometrial stromal and glandular epithelium. Normal proliferative human endometria ($n = 6$) were subjected to laser capture microdissection to separately excise stroma and glandular epithelium. The micro-dissected cells were lysed in lysis buffer and the cleared extract was electrophoresed on SDSPAGE gels prior to Western blotting with antibodies against total and phosphorylated forms of ERK1/2 kinase, ERα and antibody to β actin. Western blot signals were digitally quantitated using Kodak Image Station. Quantitated signals are plotted in (A–C). Representative Western blots are shown in (D) (42 and 44 kDa and 66 kDa for ERK1/2 and ERα, respectively). Values shown are ±standard error of the mean.

estrogenic proliferative action in glandular epithelium [2,4,5,8,12].

Estrogen is a well-known proliferative stimulus for glandular epithelial cells in the endometrium under normal and pathological conditions. Estrogen action is mediated by ERα and ERβ [11,25]. After binding to estrogen or selective estrogen receptor modulators (SERMs) such as tamoxifen, ERα is targeted to the nucleus where it regulates transcription of estrogen responsive genes [1,28]. As stated above, the ER and ERK1/2 signaling pathways crosstalk to one another, hence it is important to understand the relative expression levels of both proteins within the same tissue compartments. To the best of our knowledge, no study has attempted to quantitatively compare the expression of ER and ERK1/2 in well-separated different uterine compartments such as glandular epithelium versus stroma despite the intimate interaction between the ER and MAPK pathways. This study has performed a quantitative comparison of the expression of these proteins between endometrial stroma and epithelium and found that ER, total and active ERK1/2 kinase levels are comparable in benign, proliferative phase endometrial stroma and epithelium. Future studies may further compare expression of kinases and assess the significance of the stromal and epithelial kinase activity during the menstrual cycle and in benign and malignant endometrium with regard to tumor growth and ER action.

Conflict of interest

We have no conflict of interest to declare.

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Further reading

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